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PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(b)(2)

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TITLE OF THE INVENTION (280 CHARACTERS MAX.)

A New Ligand-Pseudoreceptor System for the Generation of Adenoviral Vectors with Altered Tropism

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ENCLOSED APPLICATION PARTS (*Check all that Apply*)

X	*Specification*	*Number of pages*	13		*Small Entity Statement*	
X	*Drawing(s)*	*Number of Sheets*	4		*Other (specify)*	

METHOD OF PAYMENT (*Check One*)

	A check or money order is enclosed to cover the Provisional filing fees	*Provisional Filing Fee Amount ($)*	$ 160.00
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Date: 24 October 2003

Respectfully submitted,

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Enclosures
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____ Additional inventors are being named a separately numbered sheets attached hereto.

PROVISIONAL APPLICATION FILING ONLY

A New Ligand-Pseudoreceptor System for the Generation of Adenoviral Vectors with Altered Tropism

BACKGROUND OF THE INVENTION

Adenoviruses (Ad) are able to infect a variety of cell types, but their wide tropism is a limitation for certain applications such as cancer therapy, because both the normal and diseased cells are transduced. The unspecific transduction has not only the negative effect on normal cell function, but also decreases the amount of therapeutic viruses delivered to the diseased cells. Therefore, targeted vectors have been developed in order to selectively localize gene expression to the tissue of interest.

Uptake of the Ad vectors (AdV) derived from serotypes 2 and 5 is a two-stage process, which involves an initial interaction of the viral fiber protein with cellular receptors CAR (coxsackievirus and Ad receptor) [1] [2]. The CAR binding domain is localized on the knob region of fiber [3]. Ad binding is then followed by the internalization of the virus, which is mediated by the interaction of the RGD motif of the penton base (viral protein) with secondary cellular receptors identified as αv integrins. This step allows virus internalization via receptor-mediated endocytosis [4]. Based on the virus entry mechanisms, several strategies were developed to create new CAR-independent entry pathway for the re-targeting of AdVs.

Several studies were undertaken using either chimeric fibers or exchanging fibers from different serotypes as a simple way to alter AdV tropism [5] since it was suggested that they might recognize different receptors and consequently have different tropism. However, true targeting of AdVs requires the ablation of the vector interaction with their natural receptors, as well as the redirection of the vector to another type of receptor, which is specific to the target cells. Mutagenesis of the fiber has been done to ablate virus-CAR interaction *in vitro*. Substitutions within knob region of fiber dramatically reduce the transduction of various CAR-positive cell lines [6] [7] [8] [9]. The shaft domain of the fiber has also been changed to modify Ad natural tropism. It was shown that the high transduction efficiency of the liver and the spleen was dramatically reduced

by the replacement of a shorter shaft within the fiber [10]. On the other hand, in order to redirect AdVs to target cells, viral capsid proteins (fiber, penton base, and hexon) were genetically modified by insertion of new ligands or chemically combined with ligands of specific receptors. Ligands such as poly-lysine, RGD motif, NGR peptides, epithelium growth factor (EGF) and gastrin releasing peptide (GRP), respectively targeting heparan sulfates, integrins, aminopeptidase N (CD13), EGF and GRP receptors, have been evaluated for their capacity to alter viral tropism [4] [11] [12] [13] [14]. Recently, a synthetic 33-amino-acid immunoglobulin G (IgG)-binding domain derived from staphylococcal protein A was inserted into the Ad fiber making possible a directed gene transfer to a wide variety of cell types by simply changing the target-specific antibody [15].

As a result of the ablation of binding to its native receptors, AdV can no longer be produced in the current complementing cell lines; hence the need for new packaging cells. One approach is to construct cell lines expressing an alternate pseudoreceptor, which allows the binding and uptake of targeted vectors. Thus, in addition to the targeting ligand incorporated into the AdV capsid for cell-specific transduction, another pseudoreceptor-binding ligand should also be inserted in the vector for their entry and propagation in packaging cells. This pair of *de novo* designed pseudoreceptor-ligand would be completely artificial, such that no natural receptors could be used for entry of the vector through the new ligand *in vivo*. For example, a cell line expressing the pseudoreceptor made of a membrane-anchored single-chain antibody against hemagglutinin (HA) was shown to be able to support HA-tagged AdV production [16]. Another cell line expressing the pseudoreceptor, which contains an anti-His sFv, allowed the infection of AdV carrying histidine-incorporated fiber [17].

The overall strategy for the development of Ad vectors (AdV) for the delivery of transgenes in specific tissues relies both on the ablation of Ad native tropism and the introduction of new tropism for target cells. In the process, AdVs ablated for their natural receptor interactions would be unable to grow in current cell lines. Consequently such ablated AdVs require new packaging cells for their generation.

SUMMARY OF THE INVENTION

It is an object of the invention to establish a system for the propagation of AdVs ablated in their native tropism.

According to one aspect of the invention, we have constructed two *de novo*-designed peptides (Ecoil and Kcoil), which interact with each other with high affinity, to establish a new receptor-ligand system. These peptides contain respectively 5 repeats of EVSALEK and KVSALKE sequences. We have also constructed a pseudoreceptor, comprising Ecoil fused with the transmembrane and cytoplasmic domains of EGFR. We have further constructed a new cell line (293E) expressing such pseudoreceptor, which efficiently propagates a CAR-ablated AdV containing the complementary Kcoil motif incorporated in its fiber knob (AdFK4m). The incorporation of another peptide K-coil that has high affinity for E-coil, at the C-terminal of the fiber (viral protein), allows the modified-virus to efficiently transduce the pseudoreceptor-expressing cell line. Moreover, the soluble peptide K-coil inhibited the infection of the modified-virus, indicating that the transduction of 293E by K-coil-incorporated AdVs is taken place via the interaction of E-coil and K-coil. We have further shown that virus entry is mediated in a CAR-independent pathway via Ecoil/Kcoil interaction. These results demonstrate that the packaging cell line 293E and AdFK4m constitute a useful platform for the generation of re-targeted AdVs.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is a schematic diagram of the EGFR-Ecoil pseudoreceptor. The EGFR signal sequence directs the receptor to the cell surface and the EGFR transmembrane domain anchors the receptor in the plasma membrane. The 6 His permit protein detection by immunoblot and flow cytometry.

Figure 2 is a Western Blot analysis of the EGFR-Ecoil pseudoreceptor expression. Transiently transfected 293 cells lysates were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with an anti-his antibody (lanes1 and 2) and anti-EGFR antibody (lanes 3 and 4).

Figure 3 illustrates an Analysis of surface expression of the EGFR-Ecoil pseudoreceptor in 293E cells. Cells were detached by cell dissociation solution (sigma), resuspended at $1x10^6$ cells/ml, and incubated with 10ul of anti-his Ab, followed by the incubation of 6ul of anti-mouse Ab*. The fluorescence intensity is plotted on a logarithm scale on the x-axis. The empty peak represent 293 cells while the shadowed peak represent 293E cells.

Figure 4 illustrates the growth profile of 293E. Cells were seeded at $2x10^5$ in DMEM medium supplemented with 10% of heat-inactivated fetal bovine serum, and counted on a daily basis until the monlayers reach confluency.

Figure 5 is a Western Blot illustrating the analysis of fiber-Kcoil expression. Transiently transfected 293 cells lysates in either denaturing (lanes 1, 2, 4, 6, 8 and 10) or non-denaturing conditions (lanes 3, 5, 7 and 9) were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with anti-fiber antibody at dilution of 1:500. Cells were transfected with pAd-CMV-GFP control plasmid (lane 1), or plasmids expressing wt fiber (lanes 2 and 3), fiber/K3 (lanes 4 and 5), fiber-K4 (lanes 6 and 7) and fiber-K5 (lanes 8 and 9).

Figure 6 A and B are graphs illustrating the gene transfer profile of AdFK4m/GFP. (A) 293E and 293 cells were infected with equal amounts of virus particles as indicated and GFP expression analyzed by flow cytometry. (B) 293 and 293E cells were infected with AdFK4m/GFP at a MOI of 1 and incubated with 0, 1 or 5□g of Kcoil soluble peptide. GFP expression was monitored by flow cytometry analysis at 72h pi; Black bar: 293 cells; Grey bar: 293E cells.

Figure 7 illustrates the growth kinetic of Ad/GFP and AdFK4m/GFP in cells 293E. On day 0, 293E cells were infected with Ad/GFP or AdFk4m/GFP at MOI of 5. At days 1, 2, or 3 post-infection, the cells were harvested, freeze-thawed, and the infectious particles released were determined by measuring the GFP expression flow cytometry analysis. Titers were expressed as PFU/ml of cell lysate.

DETAILED DESCRIPTION OF THE INVENTION

METHODS AND MATERIALS

Plasmids

pMPG-EGFR-EcoiI: The EGFR signal sequence was amplified by PCR with the primers 5'-ATAAGAATGCGGCCGCATGCGACCCTCCGGGACG-3' and 5'-GGACTAG TCTTTTCCTCCAGAGCCCG-3', which allowed the insertion of a NotI site at the 5' terminus and SpeI site at the 3' terminus. 6 His and EcoiI sequence were amplified by PCR using the primers 5'-CTAGCTAGC CATCACCACCATCATCAC-3' and 5'-CCGCTCGAGTGATCCTCCACC-3' with the insertion of NheI site at the 5' terminus and XhoI site at the 3' terminus. The transmembrane and cytoplasmic parts of EGFR were amplified with the primers 5'-CCGCTCGAGCCGTCCATCGCCACTGGG-3' and 5'-CGGATATCTCATGCTC CAATAAATTC-3' with the insertion of XhoI site at the 5' terminus and EcoRV site at the 3' terminus. The three fragments were cut with appropriate enzymes and ligated, then inserted into NotI and EcoRV sites of the vector pMPG, which express both BFP and hygromycin-resistant genes from independent cassettes.

CMV-FBK3/K4/K5: The oligonucleotides 5'-GGATCTGGATCAGGTTCAG GAGTGGATCC-3' containing a linker of 5 gly-ser and BamHI site were inserted at the C terminus of the fiber gene under the control of CMV5 promoter in pCMV-FB-BFPq plasmid. KcoiI sequences were amplified with the primers 5'-CGCGGATCCAAGGTATCCGCTTTAAAG-3' and 5' CGCGGATCCCAATTGTTACTCCTTCAGAGCACT-3' (for K3), or 5'-CGGGATCCCAATTGTTATTCCTTCAAGGCTGACAC-3' (for K4), or 5'-CGGGATCCCAATTGTTACTCTTTAAGTGCTGA-3' (for K5), digested by BamHI, then inserted in BamHI site of pCMV-FB-BFPq. A MunI site was incorporated in the amplified KcoiI sequences after the stop codon.

CMV-FBK4m: A quikchange site-directed mutagenesis kit (Stratagene) was used for the mutation of fiber at aa 408. CMV-FBK4 was amplified by the primers 5'-ACCACACCAGCTCCAGAGCCTAACTGTAGACTAAATGC-3' and 5'-GCATTTAGTCTACAGTTAGGCTCTGGAGCTGGTGTGGT-3', which contain the mutation. The PCR condition is 1 cycle of 30" at 95C° and 16 cycles of 30" at 95C°, 1' at 55C° and 25' at 68C°. Then the methylated non-mutated parental DNA template was

digested by DpnI, while the mutated neo-synthesized plasmids are unmethylated, therefore uncleaved by DpnI. They were then amplified in DH5α bacterial cells after transformation.

PE4-FBk4m: The plasmid CMV-FBK4m was cut by MunI and NheI, digested fragment contains the modified part of fiber gene, it was then inserted into MunI and NheI-digested PE4 plasmid, which contains Ad sequence (84,5 mu to 100 mu) including fiber gene. The modified part of the fiber replaced the wt fiber in PE4 plasmid.

Cells

293 cells: Human embryonic kidney cells. They are maintained in condition as described previously [18].

293E cells: Stable cell line 293E was generated by transfection of 293 cells with pMPG-EGFR-Ecoil/BFPq (5μg). This transfection was done using the Polyethylenimine (PEI) (7,5μg) precipitation method. 48h post-transfection, the cells were subjected to selection for 3 weeks with hygromycin (350μg/ml). The cells expressing the highest level of BFP reporter protein were distributed into 96-well plates and expanded under the selective pressure with hygromycin.

Viruses

Viral construction

Ad/GFP AdEasy deleted in E1 and E3 regions (QBiogene) was used to produce Ad/GFP by homologous recombination with a transfer vector containing GFP gene under TR5/Cuo promoter. 100ng of AdEasy and 1μg of PmeI-linearized transfer vector were used for transformation of BJ5183 bacterial cells by electroporation (2,5KV). The resultant Ad/GFP contains the reporter GFP gene at E1 region.

AdFK4m: AdEasy deleted in E1 and E3 regions was cut with MunI, PacI and SpeI, and the digested fragments MunI/PacI and PacI/SpeI were ligated with another fragment MunI/SpeI derived from plasmid PE4-FBK4m. This later plasmid contains Ad sequence (84,5 mu to 100 mu) including fiber gene that has a mutation at aa 408 (S -> E), and a Kcoil sequence inserted at C-terminus.

AdFK4m/GFP: AdK4m was used to produce AdFK4m/GFP by homologous recombination with a transfer vector containing GFP gene under TR5/CuO promoter.

100ng of AdFK4m and 1ug of PmeI-linearized transfer vector were used for transformation of BJ5183 bacterial cells as described for Ad/GFP.

Rescue of Ad viruses 5µg of viral DNA Ad/GFP and AdFK4m/GFP were cleaved with PacI, then respectively transfected into 293E or 293 cells by PEI precipitation method. Cells were harvested after 21 days when they showed cytopathic effect. After three cycles of freeze-thawing to release Ad particles, 293E or 293 cells were infected with half of the cell lysate to propagate the viruses. The infectious titers were determined by measuring the GFP expression in 293E cells using flow cytometry (λ=525nm).

Proteins expressing analysis

Immunoblot 5µg of DNA is used for cell transfection by PEI precipitation method. 48h later, cells were collected, washed with phosphate buffered saline (PBS), lysed in 62,5mM Tris-HCl (pH6,8), 10% glycerol, 2%SDS, 2,5% 2-mercaptoethanol (denaturing condition) or non-reducing buffer (the same buffer except 1%SDS without 2-mercaptoethanol, followed by sonication. 50µg of total protein extract was loaded onto a 4-12% acrylamide gel. After transfer, the nitrocellulose membrane was blocked with PBS containing 5% dry milk, 0,1% tween 20 during 1h at room temperature, and then probed with a monoclonal antibody against histidine (1:250) (Qiagen) or fiber protein (1:500) (Neomarkers) or EGFR (1:2000) (BD Biosicences) overnight at 4°C. Proteins were then detected by using anti-mouse-peroxidase (1:5000) and the ECL chemiluminescence kit (Amersham).

Fluorescence-activated cell sorting (FACS) analysis cells were dislodged from tissue culture plate by cell dissociation solution (sigma), centrifuged at 1000rpm, and resuspended at $1x10^6$ cells/ml in complete medium containing 10% serum. Cells were incubated with 10µl antibody against to His, followed by incubation of 6µl of Alexa green fluor 488 coat anti mouse IgG (Molecular probes A-1100). Each incubation step was done during 1h on ice. The cells were analyzed by FACScan cytometer at λ of 525nm.

Virus transduction and competition essays

Cells were seeded on 6-well plates and incubated with virus at a multiplicity of infection (MOI) of 0,04; 0,4; and 4 during 3 days at 37°C For competition studies, prior

to virus infection, wells were incubated for 1h in 500ul of complete medium alone or containing Kcoil peptide (1 or 5 μg) at 37°C. The Kcoil peptide remained present during virus infection when virus was added in 500ul of medium at MOI of 1, and incubated with cells during 3 days. Transduction efficiencies were evaluated by monitoring GFP expression by flow cytometry.

RESULTS

Generation of a 293 Cell line (293E) expressing a pseudoreceptor (EGFR-Ecoil)

An artificial peptide Ecoil (5 repeats of EVSALEK) was chosen for the construction of EGFR-Ecoil pseudoreceptor. Using such an artificial ligand should exclude the possibility of accidental *in vivo* binding of a modified AdV to receptors other than the selected target. Gene encoding the fusion protein EGFR-Ecoil (Fig.1) was cloned in a mammalian expression vector pMPG under the control of a modified CMV promoter. EGFR-Ecoil comprises the signal sequence of EGFR, 6 His, Ecoil sequence, the transmembrane and cytoplasmic parts of EGFR. The EGFR signal sequence directs Ecoil to the cell surface and the EGFR transmembrane domain anchors the receptor in the plasma membrane. The 6 His permit detection of the protein by immunoblot and flow cytometry. The resultant plasmid contains also the gene for hygromycin selection and BFP (blue fluorescent protein) reporter expressed from independent cassettes. After transient transfection, the expression of EGFR-ecoil in 293 cells was confirmed by Western blotting (Fig. 2) using anti-EGFR antibody (lane 2) or anti-His antibody (lane 4). This protein is not detected in 293A cells transfected by control vector pMPG without EGFR-Ecoil gene (lanes 1 and 3).

Stable cell lines (293E) were generated by transfection of 293 cells with the plasmid pMPG-EGFR-Ecoil, followed by selection in the presence of hygromycin. The BFP positive cells were sorted using the multiwell automated cell deposition system and clonal distribution was visually checked. Five of the best clones as assessed by BFP expression were further characterized for EGFR-Ecoil pseudoreceptor surface expression by flow cytometry of cells following incubation with the anti-His Ab. Fig. 3 shows the profile of the best clone displaying a marked increase in cell fluorescence (293E) cells as compared to 293 cells (without pMPG-EGFR-Ecoil). Most of the other clones had a similar profile. The median fluorescence for 293E cells was 732, versus 11 for 293 cells

(Fig.3). This result demonstrated that the pseudoreceptors EGFR-Ecoil were displayed on the cell surface. The growth rate of the selected 293E cells was similar to that of parental cells (Fig. 4), indicating that the expression of the EGFR-Ecoil pseudoreceptor did not significantly affected the cell physiology.

Construction of AdV containing chimeric fiber incorporating Kcoil in the knob (AdF4Km/GFP)

The artificial peptide Kcoil, which has high affinity to Ecoil, was selected as the ligand to be inserted into the fiber knob of AdV. A crucial requirement for successful fiber modification by incorporation of a peptide is that this should not change its conformation nor its normal function. Two questions have been therefore addressed: Is the 35 aa segment of Kcoil small enough to be incorporated into fiber without changing its trimerization, which is essential for fiber incorporation into the capsid and proper virus assembly? If the size of the Kcoil motif was varied by eliminating 1 or 2 repeat sequences, will it be able to keep affinity to Ecoil peptide high enough to insure efficient binding of the AdV to the pseudoreceptor?

Different repeats of Ecoil and Kcoil have been synthesized and their interaction have been analyzed by BIACORE [19]. E5 (Ecoil of 5 repeats) binds K5 (Kcoil of 5 repeats) with very high affinity (Kd = 63pM). The association capacity decreased with the peptide size: Kds are 14nM and 7μM respectively for E5/K4 and E5/K3 interaction. Clearly, reducing the number of repeat by 2 in Kcoil motif dramatically decreased it's binding to Ecoil.

In our invention, we investigated the suitable repeat number in Kcoil for their incorporation into fiber without disturbing the fiber trimerization. Chimeric fiber genes containing 3, 4 or 5 repeats of Kcoil at the C-terminus were cloned in the vector pAdCMV5K7BFPq under the CMV5 promoter. A flexible linker made of 5 glycine residues was added between the fiber's last amino acid (aa) codon and first aa codon of Kcoil in order to optimize accessibility of the Kcoil in fiber to the EGFR-Ecoil pseudoreceptor. The recombinant proteins were analyzed by western blotting under denaturing (Fig 5, lanes 1, 2, 4, 6, 8 and 10) and no-denaturing conditions (lanes 3, 5, 7 and 9). FB/K3 (lane 5) and FB/K4 (lane 7) can trimerize almost at same level as wt fiber (lane 3). In contrast, the overall expression of FB/K5 (lane 9) was dramatically decreased while its trimerization was slightly affected (data not shown). Note that the anti-fiber antibody used in this western blot preferentially recognized the trimeric fiber. This result

shows that, both 3 and 4 repeats of Kcoil incorporated in fiber did not compromise the expression nor the trimerization of these proteins.

Given its higher affinity for Ecoil, we selected K4 as the ideal candidate to be inserted into virus capsid. An AdV was then constructed in which the fiber gene contained K4 at C terminus in addition to a mutation (S -> G) at aa 408 known to abolish the fiber interaction with CAR. This recombinant virus has also a reporter gene encoding for GFP at E1 region (AdK4m/GFP). The viral DNA generated in *E. coli* was transfected in 293E cells to produce the virus. A control virus Ad/GFP that contains wt fiber and GFP under the same promoter was also constructed.

Transduction of 293E cells by AdF4Km/GFP

In order to test whether the membrane-anchored EGFR-Ecoil could serve as an artificial receptor for AdFk4m/GFP, both 293 cells and 293E cells were infected with this virus at MOI of 0,04; 0,4 or 4. Transduction efficiencies were evaluated three days later by measuring GFP expression in infected cells using flow cytometry. As shown in Fig. 6 (A), 293 cells without the pseudoreceptor is barely transduced by AdFK4m/GFP. This result is consistent with the expected reduced transduction efficiency of AdV with the fiber mutation at aa 408. By contrast, the transduction efficiency in 293E cells was increased 24-fold at MOI 0,4, and 11-fold at MOI 4, indicating that AdFK4m infect 293E cells via a CAR-independent cell entry pathway.

Competitive inhibition assays were performed in order to confirm that 293E transduction by AdF4Km/GFP required the specific binding of the vector to the pseudoreceptor via Ecoil-Kcoil interaction. Soluble peptides Kcoil (1µg or 5µg) were incubated with cells 1h prior virus infection. Virus-mediated GFP gene transfer in 293E cells was inhibited by 87% (Fig 5, B). No effect was observed for the transduction of 293 cells. These results demonstrated that binding of AdFK4m/GFP to the pseudoreceptor via Ecoil-Kcoil interaction mediates virus infection to 293E cells in the absence of fiber-CAR interaction.

In conclusion, these data demonstrated that both the complementary components comprising modified Ad virion and cell line together constitute a novel system that permits the fiber receptor-independent propagation of tropism-modified AdVs.

Characterization of virus growth kinetics

Virus growth rate of the modified virus AdFK4m/GFP were tested in comparison with Ad/GFP (Fig. 7). 293E cells were infected at an MOI of 5 active virus particles/cell with both virus, and the titers were determined by measuring GFP expression by flow cytometry at 1, 2, and 3 days post-infection. The growth curves for both virus showed similar shapes and no lag was observed in recombinant virus growth. However, the production of infectious particles by the fiber-modified virus was lower than the virus with wt fiber. This could be due to suboptimal level of expression of the EGFR-Ecoil pseudoreceptor in 293E cells or to suboptimal expression of K4-fiber in AdFK4m/GFP.

In conclusion, the complementary components comprising modified Ad virion and cell line together constitute a novel system that permits the fiber receptor-independent propagation of tropism-modified AdVs. One of the main advantages of this system is the possibility of re-targeting, either through direct incorporation of ligands in the capsid, or through the construction of adapters (coil-fused ligands).

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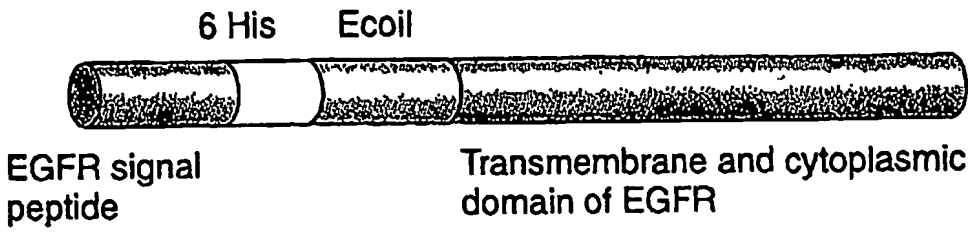


Figure 1.

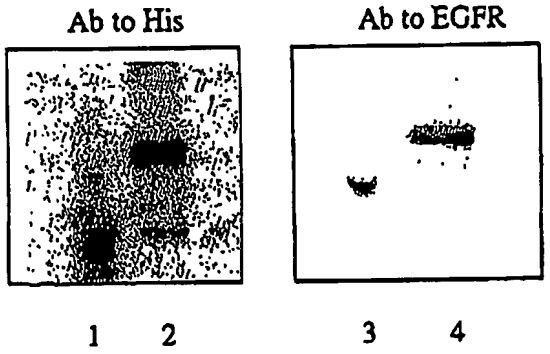


Figure 2.

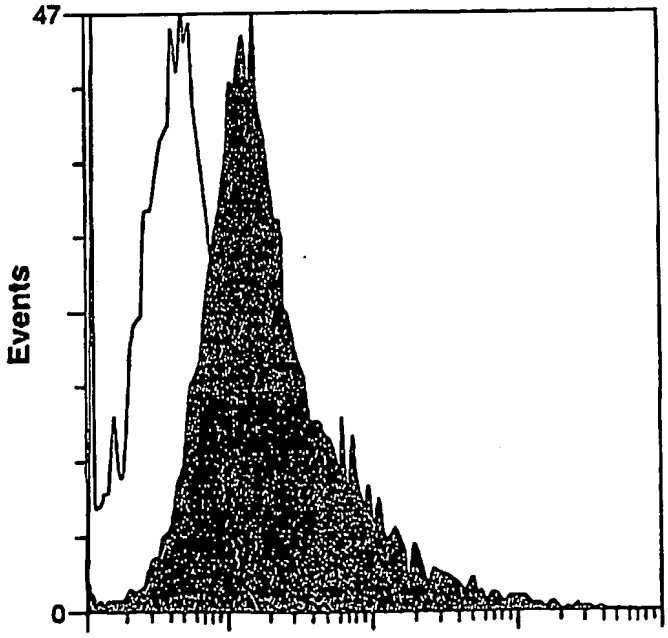


Figure 3.

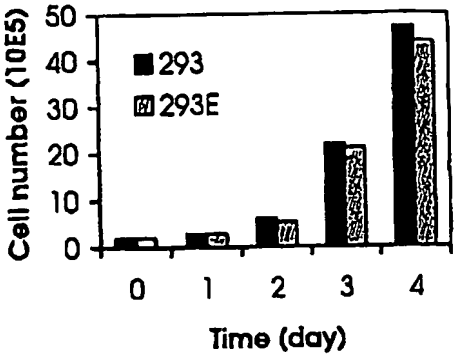


Figure 4.

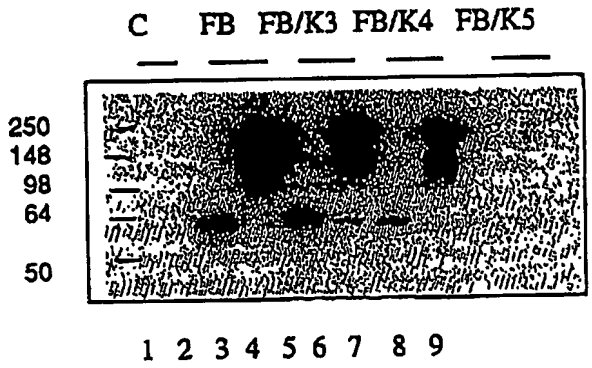
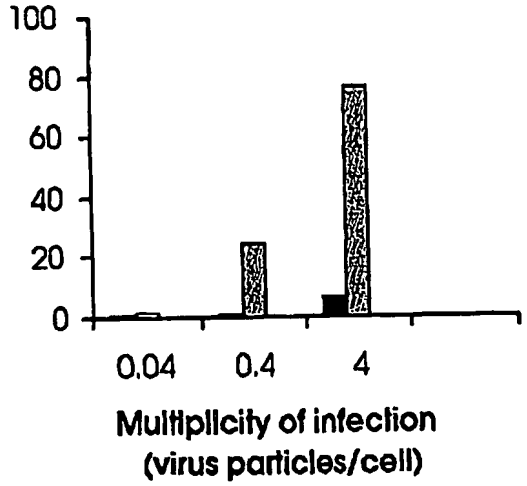


Figure 5.

A


100
80
60
40
20
0
0.04
0.4
4
Multiplicity of infection
(virus particles/cell)

B

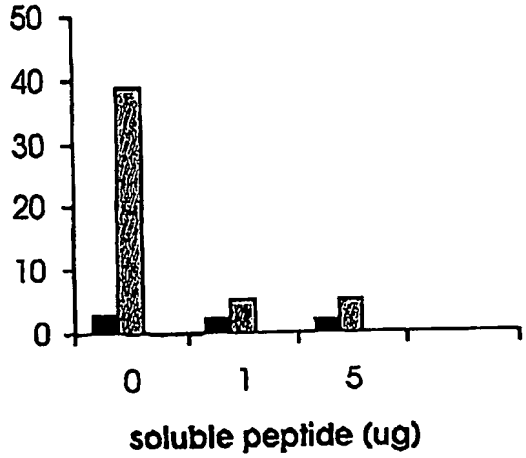

50
40
30
20
10
0
0
1
5
soluble peptide (ug)

Fig.6

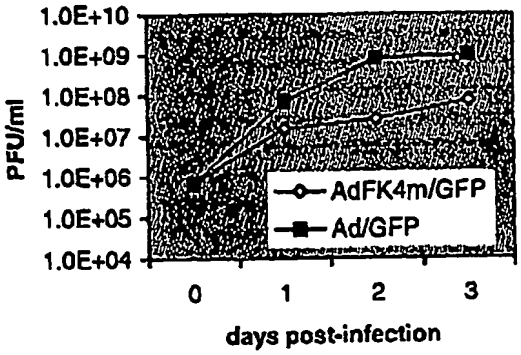

1.0E+10
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
PFU/ml
AdFK4m/GFP
Ad/GFP
0
1
2
3
days post-infection

Fig. 7

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